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Research Note

Genetic diversity of endangered *Polyporus umbellatus* from China assessed using a sequence-related amplified polymorphism technique

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ABSTRACT. *Polyporus umbellatus* (Pers.) Fries is an endangered medicinal fungus in China with *in vivo* anticancer activity, but its genetic information is lacking. Eight natural *P. umbellatus* strains collected from 7 provinces in China were subjected to sequence-related amplified polymorphism markers to estimate the level and pattern of genetic diversity. Forty-nine primer combinations generated 1219 highly reproducible and discernible loci, among which 1023 were polymorphic. The percentage of polymorphism varied from 35.71 to 96.30 with an average of 83.92. Genetic identity among all strains ranged from 0.15 to 0.78 with an average of 0.46. The unweighted pair group method with arithmetic mean dendrogram clustered 8 strains into 3 clusters, and the clustering pattern showed 3 groups. Principal coordinate analysis further indicated that the genetic diversity of *P. umbellatus* strains was unevenly distributed and instead displayed a

clustered distribution pattern. A relatively high level of genetic diversity was maintained in 8 natural *P. umbellatus* strains, but its abundance might be subjected to environmental heterogeneity, and the population structure of co-evolved *Armillaria* species may be selected by nature under the specific microenvironment.

Key words: Conservation; Chuling; Genetic diversity; SRAP; *Polyporus umbellatus*

INTRODUCTION

Chuling [*Polyporus umbellatus* (Pers.) Fries], a medicinal fungus belonging to Polyporaceae, Basidiomycetes, is widely distributed in China, Japan, Europe, and North America (Li, 2008). Its dried sclerotium has been used as a diuretic in traditional Chinese medicine for 2500 years (Yuan et al., 2004). Recently, the sclerotium has been reported to exhibit other pharmacological functions, for example, *in vivo* anticancer activity (Zhao et al., 2010). These potent pharmacological properties have attracted worldwide interest in developing this medicinal fungus; however, the over-digging of wild materials has drastically decreased its natural resources in China (Li, 2008). In the past, wild chuling was widely distributed in 13 provinces of China, including Shaanxi, Yunnan, and Hebei (Xu, 1997), but its distribution has shrunken severely, and the fungus is currently listed as an endangered species in the China Red Book (Li, 2008).

Molecular investigations on this fungus are very limited, and the genetic background of its natural strain is lacking description. Assessment of the genetic diversity prevalent in wild resources needs immediate attention (Lakhanpaul et al., 2003). Knowledge of the extent and distribution of genetic variation within a species is fundamental for evolutionary comprehension, conservation of genetic resources, and subsequent breeding strategies (Ayana et al., 2000).

DNA markers can aid in performing a quick study of the genetic diversity of a species, and sequence-related amplified polymorphism (SRAP), a new molecular marker first introduced by Li and Quiros (2001), has several advantages such as simplicity, reproducibility, and easy isolation of fragments for sequencing. This technique amplifies open reading frames, and its power for revealing genetic diversity was found highest among 4 marker systems (SRAP, simple sequence repeat, inter-simple sequence repeat, and random amplified polymorphic DNA) in buffalo grass (Budak et al., 2004). It has been widely applied in genetic diversity analyses, genetic linkage map construction, and molecular identification (Budak et al., 2004; Ren et al., 2010; Wang et al., 2011).

To conserve the natural genetic resources of this medicinal fungus as soon as possible, the mycelium growth and polysaccharide content of 7 natural *P. umbellatus* strains collected from 7 provinces in China were evaluated (Zhang et al., 2010). This study aimed to investigate the genetic diversity of 8 *P. umbellatus* strains collected from 7 provinces in China and the efficiency of SRAP markers for providing insights on conservation and breeding strategies for chuling.

MATERIAL AND METHODS

Sclerotium material

Eight natural sclerotium strains of *P. umbellatus* were collected from 7 provinces in

China (Table 1). They were identified and preserved at a local laboratory in each region after collection.

Table 1. Eight natural sclerotium strains of *Polyporus umbellatus* collected from 7 provinces in China.

Sclerotium strain	Origin	Collected by
1	Fengxian, Shaanxi Province	Our laboratory
2	Lueyang, Shaanxi Province	Our laboratory
3	Funiu Mountain, Henan Province	Research Institute of Biology, Henan Academy of Sciences
4	Changbai Mountain, Heilongjiang Province	Heilongjiang Institute of Edible Fungi
5	Daba Mountain, Sichuan Province	Mianyang Institute of Edible Fungi
6	Jizu Mountain, Yunnan Province	Yunnan Academy of Agricultural Sciences
7	Baishi Mountain, Hebei Province	Research Institute of Microbiology, Hebei Academy of Sciences
8	Shennongjia Mountain, Hubei Province	Laboratory of Fungus, Huazhong Agricultural University

DNA extraction

Activation culture of 8 original sclerotium strains and liquid culture was performed following the protocols of Zhang et al. (2010). Activated mycelium on potato dextrose agar medium was transplanted into liquid medium and cultivated at 25°C for 7 days in the dark. The mycelium was washed with double-distilled water and filtered with sterile gauze and then stored with sterile filter paper at -20°C for use after water absorption. Total genomic DNA was extracted from 100 mg fresh mycelium using an improved cetyltrimethylammonium bromide method (Zhang et al., 2011). The integrity and quality of isolated DNA was evaluated via electrophoresis on 0.7% agarose gel.

SRAP analysis

SRAP analysis was carried out based on the protocols published by Li and Quiros (2001). Eighty-one SRAP primer combinations (Table 2) were initially screened, of which 49 produced consistent and clear polymorphic fragments (Table 3). All samples were amplified 3 times. The 25-μL polymerase chain reaction (PCR) mixture contained 20 ng DNA template, 0.4 μM forward primer, 0.4 μM reverse primer, and 1X Taq MasterMix (Kangwei, Beijing). PCR amplification was performed in a PTC-200 Programmable Thermal Controller (MJ Research, Inc., Watertown, USA) with a PCR program provided by Li and Quiros (2001). SRAP fragments were separated via polyacrylamide gel electrophoresis on a DNA Analyzer Sequencer (ABI 3730XL, USA) using 4% (v/v) denaturing polyacrylamide gels.

Table 2. The forward and reverse sequence-related amplified polymorphism primers employed to obtain primer combinations.

Forward primer	Reverse primer
Me1: TGAGTCCAAACCGGATA	Em1: GACTGCGTACGAATTAAT
Me2: TGAGTCCAAACCGGAGC	Em2: GACTGCGTACGAATTTGC
Me3: TGAGTCCAAACCGGAAT	Em3: GACTGCGTACGAATTGAC
Me4: TGAGTCCAAACCGGACC	Em4: GACTGCGTACGAATTTGA
Me5: TGAGTCCAAACCGGAAG	Em5: GACTGCGTACGAATTAAC
Me6: TGAGTCCAAACCGGTAG	Em6: GACTGCGTACGAATTGCA
Me7: TGAGTCCAAACCGGTTG	Em7: GACTGCGTACGAATTATG
Me8: TGAGTCCAAACCGGATT	Em8: GACTGCGTACGAATTCGA
Me9: TGAGTCCAAACCGGTGT	Em9: GACTGCGTACGAATTAGC

Data analysis

Band positions for each mycelium and primer combination were scored as either present (1) or absent (0). The scores were entered into a database program (Microsoft Excel) and compiled in a binary matrix for phonetic analysis using NTSYS-pc System version 2.1 (Rohlf, 2000). The SIMQUAL program was used to calculate the Jaccard similarity coefficient, and a graphic phenogram (dendrogram) of the genetic relatedness among 8 strains was produced by means of the unweighted pair group method with arithmetic mean (UPGMA) analysis.

The binary data for strains were subjected to principal coordinate analysis (PCA) (Peakal and Smouse, 2001), and the first 2 principal coordinates were plotted to indicate the multilateral genetic relationships among the chuling accessions.

RESULTS

Products of SRAP amplification

A total of 1219 bands were scored from PCR amplification of 8 chuling genomic DNAs using the 49 selected SRAP primer combinations (see Table 3) with an average of 24.88 bands per primer combination. All 1023 bands were polymorphic, with a percentage of 83.92%. The average number of polymorphic bands varied from 8 (Me3/Em1) to 37 (Me4/Em3) per primer combination, with a mean of 20.88. The percentage of polymorphic bands produced by each primer pair ranged from 35.71% (Me4/Em8) to 96.30% (Me8/Em3), with an average of 83.92% per primer combination.

Genetic diversity of chuling strains

The percentage of polymorphism for each strain is shown in Table 4 and ranged from 67.17% (No. 7 from Hebei Province) to 74.55% (No. 5 from Sichuan Province). Jaccard genetic similarity coefficients between all pairs of the 8 strains are listed in Table 5. Genetic identity among 8 *P. umbellatus* strains ranged from 0.15 to 0.78, with an average of 0.46. Among them, No. 2 (from Shaanxi Province) shared the least similarity with No. 7 (from Hebei Province) (0.1487) and No. 4 (from Heilongjiang Province) had the greatest similarity with No. 5 (from Sichuan Province; 0.78).

To detect whether the genetic diversity of chuling strains was evenly distributed, we performed PCA based on the data matrix from the DNA amplification products. The analysis of genetic similarity between pairwise strains also revealed considerable genetic diversity. The scatterplots based on the first 2 principal coordinates accounted for 67.66% of the total variation and exhibited relatively significant differentiation among 8 natural chuling strains (Figure 1). Strains No. 3 (from Henan Province) and No. 7 (from Hebei Province) showed the farthest distance among all scatter plots, which corresponded to the finding that they have the least genetic similarity (0.15 in Table 5).

The binary matrix of SRAP data was also used to construct an UPGMA tree, and relatively high genetic diversity was revealed in the 8 wild chuling strains based on Jaccard similarity coefficient values (Table 5). The dendrogram obtained from UPGMA grouped 8 strains into 3 clusters (Figure 2). Cluster I grouped 6 strains (Nos. 1, 2, 4, 5, 6,

and 8) from Shaanxi, Heilongjiang, Sichuan, Yunnan, and Hubei Provinces; No. 3 (from Henan Province) and No. 7 (from Hebei Province) were included in clusters II and III, respectively. This distribution pattern agrees with the result of PCA analysis shown in Figure 1. In addition, none of the clusters in the dendrogram showed any strict relationship with geographical distribution.

Table 3. Forty-nine selected sequence-related amplified polymorphism primer combinations and the percentage of polymorphism obtained from 8 natural *Polyporus umbellatus* strains in China.

Primer combination	Total bands	Polymorphic bands	Percentage of polymorphic bands
Me1/Em4	29	24	85.29%
Me1/Em5	23	21	91.30%
Me1/Em6	14	10	71.43%
Me1/Em7	32	24	75.00%
Me1/Em8	32	25	78.13%
Me1/Em9	15	12	80.00%
Me2/Em1	12	10	83.33%
Me2/Em6	41	36	87.80%
Me3/Em1	10	8	80.00%
Me3/Em2	21	17	80.95%
Me3/Em4	20	16	80.00%
Me3/Em6	18	14	77.78%
Me3/Em7	15	11	73.33%
Me3/Em8	15	13	86.67%
Me4/Em1	13	9	69.23%
Me4/Em2	28	20	71.43%
Me4/Em3	41	37	90.24%
Me4/Em4	18	14	77.78%
Me4/Em5	18	16	88.89%
Me4/Em6	19	18	94.74%
Me4/Em7	27	19	70.37%
Me4/Em8	28	10	35.71%
Me4/Em9	28	23	82.14%
Me5/Em1	15	12	80.00%
Me5/Em2	20	18	90.00%
Me5/Em3	34	31	91.18%
Me5/Em4	27	20	74.07%
Me5/Em5	12	11	91.67%
Me5/Em6	35	28	80.00%
Me5/Em7	24	22	91.67%
Me6/Em2	27	25	92.59%
Me6/Em3	25	21	84.00%
Me6/Em4	32	29	90.63%
Me6/Em5	37	34	91.89%
Me6/Em6	37	34	91.89%
Me6/Em8	39	31	79.49%
Me7/Em1	23	20	86.96%
Me7/Em2	31	25	80.65%
Me7/Em3	27	20	74.07%
Me7/Em4	28	26	92.86%
Me8/Em3	27	26	96.30%
Me8/Em4	27	25	92.59%
Me8/Em7	25	23	92.00%
Me8/Em9	21	20	95.24%
Me9/Em1	19	17	89.47%
Me9/Em4	22	17	77.27%
Me9/Em5	31	29	93.55%
Me9/Em6	25	22	88.00%
Me9/Em8	32	30	93.75%
Total	1219	1023	83.92%
Average	24.88	20.88	83.92%

Table 4. Percentage of polymorphism obtained from 8 natural *Polyporus umbellatus* strains in China by using 49 selected sequence-related amplified polymorphism primer combinations.

Sclerotium strain[a]	Total bands	Polymorphic bands	Percentage of polymorphic bands
1	704	508	72.16%
2	715	519	72.59%
3	671	475	70.79%
4	758	562	74.14%
5	770	574	74.55%
6	724	528	72.93%
7	597	401	67.17%
8	751	555	73.90%

[a]The strain code is the same as in Table 1.

Table 5. Jaccard genetic idendity of 8 natural *Polyporus umbellatus* strains collected seven provinces of China.

Sclerotium strain[a]	1	2	3	4	5	6	7	8
1	1							
2	0.66	1						
3	0.48	0.47	1					
4	0.53	0.57	0.44	1				
5	0.52	0.56	0.42	0.78	1			
6	0.51	0.54	0.38	0.67	0.77	1		
7	0.17	0.15	0.15	0.17	0.18	0.16	1	
8	0.51	0.55	0.39	0.68	0.69	0.68	0.20	1

[a]The strain code is the same as in Table 1.

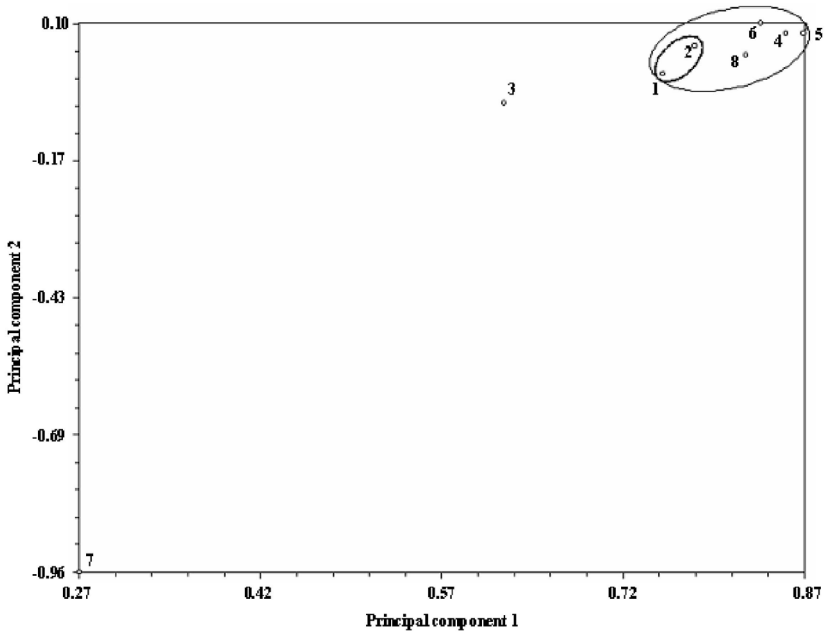

Figure 1. Principal coordinate analysis plot of the first two principal coordinates of 8 natural *Polyporus umbellatus* strains based on sequence-related amplified polymorphism markers. The number at each plot refers to the corresponding number in Table 1.

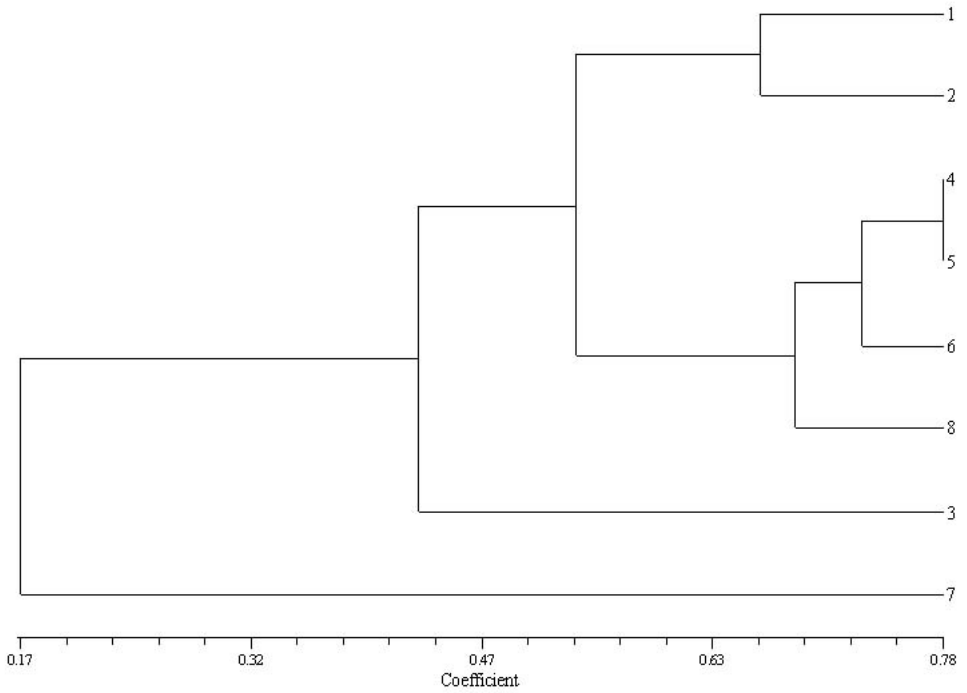

Figure 2. The dendrogram tree of 8 natural *Polyporus umbellatus* strains collected from 7 provinces in China based on Jaccard similarity coefficients by using unweighted pair group method with arithmetic mean method from sequence-related amplified polymorphism markers. The numbers correspond to those listed in Table 1.

DISCUSSION

Genetic diversity and genetic variation among chuling strains

SRAP is the first molecular marker applied to assess the level and pattern of genetic diversity in chuling strains. Our SRAP survey of 8 natural *P. umbellatus* strains indicates a relatively high level of genetic diversity, with 83.92% of bands being polymorphic. This relatively high genetic diversity may be attributed to several characteristics. Geographic isolation has played an important role during the formation of genetic diversity and genetic variation of chuling, although our result showed no strict geographic relationship among the strains (Figure 2). Another fungus, *Armillaria*, provides nutrition to chuling and often parasitizes it on *Quercus* and other broadleaf trees (Imazeki and Hongo, 1965; Li, 2008). The sequences of 5.8S recombinant DNA and the flanking internal transcribed spaces (1 and 2) of chuling and isolated *Armillaria* species have 99.36% similarity, indicating that they may represent co-evolution in the complex (Xing and Guo, 2004). Various environmental factors also affect mycelium growth in chuling (Zhang et al., 2010), which might determine its infectious capabilities. Conversely, *Armillaria* species are widely distributed throughout the world and comprise

several biological species in North America, Europe, Australia, Japan, and China (Kikuchi and Yamaji, 2010; Wang, 2010). Moreover, chuling associates with many *Armillaria* species in China and Japan (Kikuchi and Yamaji, 2010), although Xu et al. (2003) have reported that *Armillaria mellea* can form the sclerotium of chuling. Therefore, different environments may affect the composition of *Armillaria* species populations, which would form selection pressure on the genetic structure of local chuling strains.

Relationship among mycelium growth, polysaccharide content, and SRAP markers

In a previous study, we found that strains No. 1 (from Shaanxi) and No. 4 (from Heilongjiang) had the greatest dry weights of mycelium; Nos. 1 and 8 (from Hubei) had the highest polysaccharide content, and No. 8 (from Hebei) and No. 5 (from Sichuan) displayed the fastest mycelium growth (Zhang et al., 2010). The relationships among SRAP markers, mycelium growth, polysaccharide content, number of asexual spores, and calcium oxalate crystals are unobvious when comparing these indexes with the results in this study. Therefore, the level of genetic diversity for chuling strains may be subjected to environmental heterogeneity, and the population structure of *Armillaria* species may be selected by nature under the specific microenvironment.

Conservation and future outlook

Although the sclerotia of chuling can be produced using artificial infection with *Armillaria* species, asexual propagation is still the main pathway adopted on Chinese farms (Zhang et al., 2010) owing to the absence of natural sclerotia (as "seeds") and low production of artificial sclerotia. Long-term asexual propagation is well known to cause the degradation of both the seeds and the quality of medicinal materials. Out-of-order introduction among regions could mix these seed resources, which makes the degradation of *P. umbellatus* more grave and in turn results in inconstant quality of medicinal materials even among fungi produced in the same field. In contrast to the relatively high genetic diversity found among the 8 strains, many landraces located in those areas with convenient traffic are disappearing owing to uninterrupted digging by local habitants.

Loss of genetic diversity during domestication would be unavoidable, although the extensive gene flow obtained through sexual hybridization between different types could to a certain degree counteract the effects of genetic drift under the current fragmentation habitats. Future studies should include more strains of chuling and *Armillaria* species from all sites to provide germplasm resources, and further cultivar breeding should take place via sexual hybridization for the sustainable use of this medicinal fungus.

This preliminary study provides necessary genetic information for future basic and applied research efforts related to chuling. The results open the door for new opportunities in comparative genetic research in other countries.

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REFERENCES

Ayana A, Bekele E and Bryngelsson T (2000). Genetic variation in wild sorghum (*Sorghum bicolor* ssp. *verticilliflorum* (L.) Moench) germplasm from Ethiopia assessed by random amplified polymorphic DNA (RAPD). *Hereditas* 132: 249-254.

Budak H, Shearman RC, Parmaksiz I, Gaussoin RE, et al. (2004). Molecular characterization of Buffalograss germplasm using sequence-related amplified polymorphism markers. *Theor. Appl. Genet.* 108: 328-334.

Imazeki R and Hongo T (1965). Colored Illustrations of Fungi of Japan. Vol. 2. Hoikusha, Osaka.

Kikuchi G and Yamaji H (2010). Identification of *Armillaria* species associated with *Polyporus umbellatus* using ITS sequences of nuclear ribosomal DNA. *Mycoscience* 51: 366-372.

Lakhanpaul S, Velayudhan KC and Bhat KV (2003). Analysis of genetic diversity in Indian taro [*Colocasia esculenta* (L.) Schott] using random amplified polymorphic DNA (RAPD) markers. *Genet. Resour. Crop Evol.* 50: 603-609.

Li G and Quiros CF (2001). Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in *Brassica*. *Theor. Appl. Genet.* 103: 455-461.

Li SQ (2008). Endangered *Polyporus umbellatus* need immediate conservation. *Mod. Chin. Med.* 10: 43-45.

Peakall R and Smouse PE (2001). GenAlEX V5: Genetic Analysis in Microsoft Excel. Population Genetic Software for Teaching and Research. Australian National University, Canberra. Available at [http://biology.anu.edu.cn/BoZo/GenAlEX/Welcome.html] Accessed November 27, 2012.

Ren X, Huang J, Liao B, Zhang X, et al. (2010). Genomic affinities of *Arachis* genus and interspecific hybrids were revealed by SRAP markers. *Genet. Resour. Crop Evol.* 57: 903-913.

Rohlf FJ (2000). NTSYS-PC Version 2.10 s. Numerical Taxonomy and Multivariate Analysis System. Exeter Publications, Setauket.

Wang HC (2010). Advances in the studies of systematics of *Armillaria* all over the world. *J. Chongqing Univer.* 27: 61-68.

Wang Z, Wang JE, Wang XM, Gao HW, et al. (2011). Assessment of genetic diversity in *Galega officinalis* L. using ISSR and SRAP markers. *Genet. Resour. Crop Evol.* 59: 865-873.

Xing XK and Guo SX (2004). The phylogenetic relationships of *Grifola umbellata* and its companion fungus: evidence from ITS sequence analysis. *Microbiology* 31: 34-38.

Xu GB, Fu WJ and Zhao XK (2003). Advances in studies on *Polyporus umbellatus* in China. *J. Fung. Res.* 1: 58-61.

Xu JT (1997). Medicinal Fungi in China. United Publishing House of Beijing Medical University and Chinese Union Medical University, Beijing.

Yuan D, Mori J, Komatsu KI, Makino T, et al. (2004). An anti-aldosteronic diuretic component (drain dampness) in *Polyporus* sclerotium. *Biol. Pharm. Bull.* 27: 867-870.

Zhang YJ, Fan S, Liang ZS, Wang W, et al. (2010). Mycelial growth and polysaccharide content of *Polyporus umbellatus*. *J. Med. Plant Res.* 4: 1847-1852.

Zhang YJ, Qin Y, Wang Z, Guo L, et al. (2011). DNA isolation and optimization of sequence-related amplified polymorphism-polymerase chain reaction (SRAP-PCR) condition for endangered *Polyporus umbellatus*. *J. Med. Plant Res.* 5: 6890-6894.

Zhao YY, Chao X, Zhang Y, Lin RC, et al. (2010). Cytotoxic steroids from *Polyporus umbellatus*. *Planta Med.* 76: 1755-1758.

